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Several *Metarhizium* Species Produce Ergot Alkaloids in a Condition-Specific Manner

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ABSTRACT Genomic sequence data indicate that certain fungi in the genus *Metarhizium* have the capacity to produce lysergic acid-derived ergot alkaloids, but accumulation of ergot alkaloids in these fungi has not been demonstrated previously. We assayed several *Metarhizium* species grown under different conditions for accumulation of ergot alkaloids. Isolates of *M. brunneum* and *M. anisopliae* accumulated the lysergic acid amides lysergic acid α-hydroxyethyl amide, ergine, and ergonovine on sucrose-yeast extract agar but not on two other tested media. Isolates of six other *Metarhizium* species did not accumulate ergot alkaloids on sucrose-yeast extract agar. Conidia of *M. brunneum* lacked detectable ergot alkaloids, and mycelia of this fungus secreted over 80% of their ergot alkaloid yield into the culture medium. Isolates of *M. brunneum*, *M. flavoviride*, *M. robertsii*, *M. acridum*, and *M. anisopliae* produced high concentrations of ergot alkaloids in infected larvae of the model insect *Galleria mellonella*, but larvae infected with *M. pingshaense*, *M. album, M. majus*, and *M. guizhouense* lacked detectable ergot alkaloids. Alkaloid concentrations were significantly higher when insects were alive (as opposed to killed by freezing or gas) at the time of inoculation with *M. brunneum*. Roots of corn and beans were inoculated with *M. brunneum* or *M. flavoviride* and global metabolomic analyses indicated that the inoculated roots were colonized, though no ergot alkaloids were detected. The data demonstrate that several *Metarhizium* species produce ergot alkaloids of the lysergic acid amide class and that production of ergot alkaloids is tightly regulated and associated with insect colonization.

IMPORTANCE Our discovery of ergot alkaloids in fungi of the genus *Metarhizium* has agricultural and pharmaceutical implications. Ergot alkaloids produced by other fungi in the family Clavicipitaceae accumulate in forage grasses or grain crops; in this context they are considered toxins, though their presence also may deter or kill insect pests. Our data report ergot alkaloids in *Metarhizium* species and indicate a close association of ergot alkaloid accumulation with insect colonization. The lack of accumulation of alkaloids in spores of the fungi and in plants colonized by the fungi affirms the safety of using *Metarhizium* species as biocontrol agents. Ergot alkaloids produced by other fungi have been exploited to produce powerful pharmaceuticals. The class of ergot alkaloids discovered in *Metarhizium* species (lysergic acid amides) and their secretion into the growth medium make *Metarhizium* species a potential platform for future studies on ergot alkaloid synthesis and modification.

KEYWORDS ergot alkaloids, *Metarhizium*, alkaloid secretion, biocontrol agents, lysergic acid

Ergot alkaloids are a diverse family of fungus-specialized metabolites, including simple clavine ergot alkaloids and more complex lysergic acid derivatives. Lysergic acid-derived ergot alkaloids, in particular, have been very important in agriculture and

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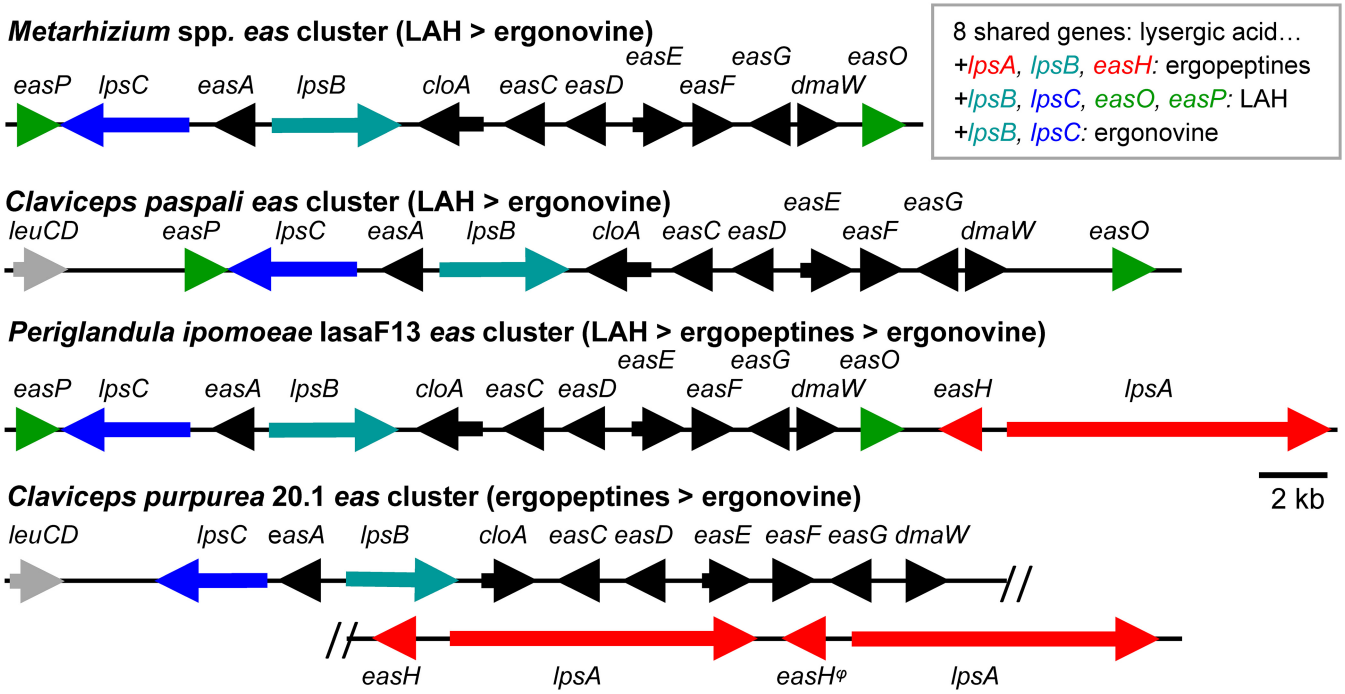

FIG 1 Ergot alkaloid synthesis (*eas*) gene clusters from several fungi. Arrows indicate genes controlling steps in the ergot alkaloid pathway. The eight genes drawn in black and common to all clusters are required to produce lysergic acid. Genes drawn in color (refer to key) are required or hypothesized to derivatize lysergic acid into amides or peptides. The major products of each cluster and their relative abundance are indicated in parentheses. The inset key (upper right) shows the association of particular genes with specific ergot alkaloids. With the exceptions of *M. majus* ARSEF 297 (37) and *M. rileyi* ARSEF 4871 (38) (described in the text), all sequenced isolates of *Metarhizium* species contain an ergot alkaloid synthesis cluster with genes in the same order and orientation as pictured. The clusters have been redrawn from data presented by Gao et al. (31) and Schardl et al. (32, 33).

in medicine. Amides and peptides containing lysergic acid have contaminated grain and forage crops resulting in human and animal toxicoses (1–4). Additionally, derivatives of lysergic acid are used to synthesize a range of pharmaceuticals used to treat dementia, migraines, Parkinson's disease, and hyperprolactinemia (5–8). Lysergic acid-derived ergot alkaloids are produced by representatives of several genera in the fungal family Clavicipitaceae, including *Claviceps* spp., *Epichloë* spp., *Balansia* spp., and *Periglandula* spp. (4, 9). Each of these fungi is closely associated with plants in symbioses that range from mutualism to parasitism (10, 11). The significance of ergot alkaloids to these plant-fungal symbioses is still being studied, but data indicate roles in deterring mammalian herbivory (12–14) and deterring or killing insect herbivores (15–17).

The ecologically important fungal genus *Metarhizium* is a member of the Clavicipitaceae. *Metarhizium* species are noteworthy because of their ability to colonize plant roots in a beneficial way (18–21) and their ability to parasitize insects. In fact, formulations of several *Metarhizium* species are used as biocontrol agents to control insect pests (22). Examples include biocontrol of spittle bug on sugar cane in Brazil by *M. anisopliae* (23), biocontrol of locusts by formulations of *M. acridum* (e.g., Green Muscle and Green Guard) in Africa and Australia (24, 25), as well as controlled trials of malaria-vectoring mosquitoes in Burkina Faso by genetically modified *M. pingshaense* (26). Significant previous research on *Metarhizium* species indicate that hydrolytic enzymes (19, 27–29) and a class of toxins called destruxins (30) are both important in pathogenesis of insect hosts.

Although *Metarhizium* species have not been demonstrated previously to contain ergot alkaloids, genomes of *M. robertsii* ARSEF 23 (an isolate originally classified as *M. anisopliae*) and *M. acridum* CQMa 102 have been observed to contain a cluster of genes homologous to those required for ergot alkaloid biosynthesis in other fungi (31) (Fig. 1). The genes involved in ergot alkaloid synthesis are found adjacent to each other in

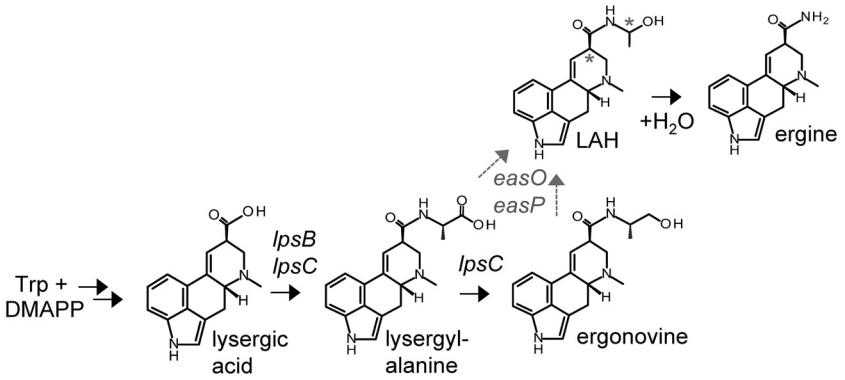

FIG 2 Pathway to synthesis of lysergic acid amides. Lysergic acid results from the activities of enzymes encoded by eight genes (*dmaW*, *easF*, *easE*, *easC*, *easD*, *easA*, *easG*, and *cloA*) (4, 9). Genes that control the modification of lysergic acid or its derivatives are indicated at relevant steps. Dashed gray arrows indicate alternate, hypothetical routes to LAH. Asterisks in the LAH structure indicate chiral carbons that yield stereoisomers as discussed in the text. Ergine arises from the spontaneous hydrolysis of LAH (42, 44, 45). Trp, L-tryptophan; DMAPP, dimethylallylpyrophosphate.

clusters (4, 9, 32, 33), and the inclusion or exclusion of certain genes provides insight into the biosynthetic capacity of the fungus. The gene clusters of *M. robertsii* and *M. acridum* resemble those of fungi that produce lysergic acid amides in having a core set of eight genes required to produce lysergic acid (shown in black in Fig. 1), in addition to genes that have been demonstrated (i.e., *lpsB* and *lpsC*) (34, 35) or hypothesized (i.e., *easO* and *easP*) (4, 9, 32) to encode enzymes involved in derivatizing lysergic acid into amide forms (Fig. 1 and 2). Genomes of six additional *Metarhizium* species have been sequenced and are publicly available (36–38), though their capacity for ergot alkaloid synthesis has not been investigated.

Considering the agricultural and pharmaceutical importance of ergot alkaloids and the presence of ergot alkaloid synthesis genes in the genomes of *Metarhizium* species associated with plants and marketed as biocontrol agents, our objective in this study was to determine the ergot alkaloid biosynthesis capacity of several *Metarhizium* species. Given the multiple niches occupied by these fungi, we analyzed them for ergot alkaloids in saprotrophic culture, in insects, and in plants by high performance liquid chromatography (HPLC) and liquid chromatography-mass spectrometry (LC-MS) approaches.

RESULTS

Ergot alkaloid synthesis gene clusters in *Metarhizium* genomes. Since the genomes of *M. robertsii* ARSEF 23 and *M. acridum* CQMa 102 had been noted to contain ergot alkaloid synthesis (*eas*) gene clusters (31), we analyzed the published genomes of *M. album* ARSEF 1941, *M. anisopliae* ARSEF 549, *M. brunneum* ARSEF 3297, *M. guizhouense* ARSEF 977 (37), and *M. anisopliae* Ma69 (36) for ergot alkaloid synthesis genes. Each of these genomes contained an *eas* gene cluster homologous to, and syntenic with, the clusters previously identified in *M. acridum* and *M. robertsii* (31) (Fig. 1). We also investigated the published genomes of *M. majus* ARSEF 297 (37) and *M. rileyi* ARSEF 4871 (38). The genome of *M. majus* contained homologs of *easO* and *easP*, as well as a partial copy of *lpsC*, but lacked homologs of several other *eas* genes, including the genes for the first nine steps of the pathway. The genome of *M. rileyi* lacked homologs of all tested *eas* genes.

***Metarhizium* spp. produce ergot alkaloids in culture in an isolate-specific manner.** Three of 10 tested isolates of *M. brunneum* accumulated the ergot alkaloids lysergic acid α-hydroxyethylamide (LAH) and its degradation product ergine, as well as lesser quantities of ergonovine, when grown on sucrose-yeast extract agar (SYE) (Fig. 3A; Table 1). These alkaloids were identified by their fluorescence properties and retention times relative to an authentic standard (for ergonovine) or standards pre-

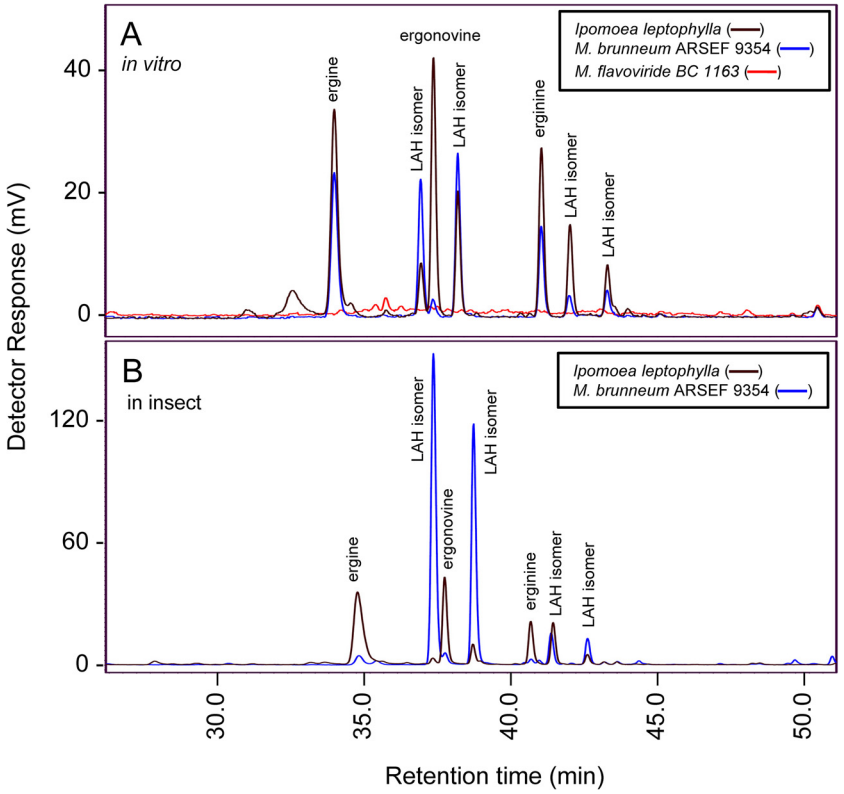

FIG 3 High performance liquid chromatography analyses of *Metarhizium* species from cultures (A) and in the insect *Galleria mellonella* (B). *Periglandula* sp.-infected *Ipomoea leptophylla* served as a reference for the indicated lysergic acid amides. Detection was by fluorescence with excitation at 310 nm and emission at 410 nm.

pared from *Periglandula* sp.-infected *Ipomoea leptophylla* (LAH and ergine) (39). LC-MS fragmentation studies supported the identity of the most abundant ergot alkaloid in *M. brunneum* cultures as LAH (Fig. S1 in the supplemental material), and accurate mass LC-MS identified each of the four stereoisomeric forms of LAH with the identical molecular formula (Fig. S2). The accumulation of ergot alkaloids in *M. brunneum* depended on the culture medium on which the fungus was grown; isolates of *M. brunneum* produced ergot alkaloids on SYE but not on corn meal agar or malt extract agar. Several additional *Metarhizium* species, including *M. acridum* (two isolates), *M. album* (three isolates), *M. anisopliae* (four isolates), *M. flavoviride* (one isolate), *M. guizhouense* (two isolates), *M. majus* (two isolates), *M. pingshaense* (one isolate), and *M. robertsii* (two isolates), were tested for accumulation of ergot alkaloids on SYE. Among these additional isolates, only *M. anisopliae* ARSEF 7426 accumulated detectable ergot alkaloids on SYE when analyzed by HPLC with fluorescence detection (Table 1).

Secretion of ergot alkaloids from *M. brunneum*. When grown on SYE broth, *M. brunneum* ARSEF 9354 secreted the majority of its ergot alkaloids into the culture medium. Culture fluids and fungal mycelia and conidia were harvested, separated, and measured after 7 days. Ergot alkaloids were quantified from solid and liquid fractions and expressed on a total culture basis. The proportion of alkaloids secreted into the medium was 0.78, 0.85, and 0.84 for ergine, LAH, and ergonovine, respectively (Fig. 4). The proportions secreted are more than seven times greater than the proportion of ergot alkaloids secreted from another ergot alkaloid-producing fungus, *Neosartorya fumigata* (syn. *Aspergillus fumigatus*) (40).

Ergot alkaloids in *Metarhizium brunneum* are not associated with conidia. Since conidia of the fungus *N. fumigata* have been documented to contain high concentra-

TABLE 1 Accumulation of ergot alkaloids from isolates of *Metarhizium* species colonizing sucrose-yeast extract agar (SYE culture) or larvae of *Galleria mellonella* (insect)

Species and isolate	Origin	SYE culture	Insect
M. acridum ARSEF 324	Orthoptera	–[a]	LAH[b]
M. acridum ARSEF 7486	Orthoptera	–	LAH
M. album ARSEF 1941[c]	Hemiptera	–	–
M. album ARSEF 2082	Hemiptera	–	–
M. album ARSEF 2176	Hemiptera	–	–
M. anisopliae ARSEF 1080	Lepidoptera	–	LAH
M. anisopliae ARSEF 7426	Diptera	LAH	n.t.[d]
M. anisopliae ARSEF 7487	Orthoptera	–	LAH
M. anisopliae BC 1203	Diplopoda	–	LAH
M. brunneum ARSEF 1116	Lepidoptera	LAH	LAH
M. brunneum ARSEF 2042	Soil	–	LAH
M. brunneum ARSEF 2107	Coleoptera	–	–
M. brunneum ARSEF 3826	Diptera	LAH	LAH
M. brunneum ARSEF 5635	Orthoptera	–	LAH
M. brunneum ARSEF 6392	Coleoptera	–	LAH
M. brunneum ARSEF 7434	Unknown	–	LAH
M. brunneum ARSEF 8515	Blattodea	–	LAH
M. brunneum ARSEF 8534	Blattodea	–	LAH
M. brunneum ARSEF 9354	Soil	LAH	LAH
M. flavoviride BC 1163	Diplopoda	–	LAH
M. guizhouense ARSEF 6238	Lepidoptera	–	–
M. guizhouense CBS 258.90	Lepidoptera	–	–
M. majus ARSEF 1914	Coleoptera	–	–
M. majus ARSEF 1946	Coleoptera	–	–
M. pingshaense ARSEF 5197	Coleoptera	–	–
M. robertsii ARSEF 23[c]	Coleoptera	–	LAH
M. robertsii ARSEF 2575	Coleoptera	–	LAH

[a]–, no ergot alkaloids detected.
[b]LAH, ergot alkaloids produced, with lysergic acid α-hydroxyethylamide being the most abundant.
[c]Isolate with sequenced genome.
[d]n.t., not tested.

tions of ergot alkaloids (41), and the conidia of *Metarhizium* species are produced and dispersed at very high concentrations (e.g., 5×10^{10} conidia/g in commercial product, applied at a rate of 4.6×10^{10} conidia/m^2) (US-EPA registration number 70127-10; https://www3.epa.gov/pesticides/chem_search/ppls/070127-00010-20130116.pdf, accessed January 29, 2020) for the purpose of insect biocontrol, it is important to understand whether conidia contain ergot alkaloids that may present a threat to applicator or producer health. To test whether ergot alkaloids accumulated in *M. brunneum* conidia, conidia from cultures on SYE were collected independently of mycelia and growth medium by inverting the culture dish and collecting conidia on the inside of the dish's lid and suspending them in methanol. No ergot alkaloids were detected in three separate samples of *M. brunneum* conidia weighing 10 mg, 14 mg, or 15 mg. The fluorescence HPLC method employed in these assays has a limit of detection of 5 fmol per mg of tissue. The corresponding cultures (containing mycelia of

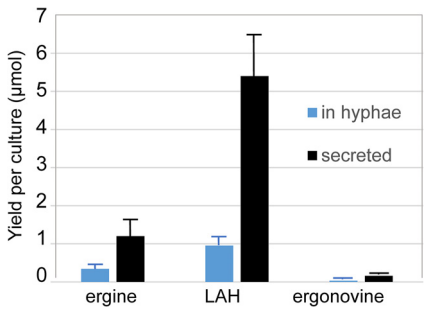

FIG 4 Mean accumulation of ergot alkaloids in hyphae versus growth medium of cultures of *M. brunneum*. Error bars represent standard error of the mean ($n = 5$).

the fungus embedded in colonized SYE medium) were checked and found to contain LAH at a mean concentration of 0.8 μM ($\pm$ 0.1 μM SE), which corresponds to approximately 300 pmol per SYE culture volume required to yield 10 mg of conidia.

Multiple *Metarhizium* species produce ergot alkaloids in inoculated insects. *Metarhizium brunneum* ARSEF 9354 was studied extensively for its ability to produce ergot alkaloids in the model insect *Galleria mellonella*, and isolates of several other species were also screened in *G. mellonella* for ergot alkaloids. In alkaloid extracts obtained from *M. brunneum* ARSEF 9354-infected larvae, we observed stereoisomers of LAH eluting at 37 min and 38 min to be highly abundant in fresh extracts relative to the 41- and 43-min isomers (Fig. 3B). The isomers of LAH eluting at 37 min and 38 min remained in approximately equal proportions relative to each other over time (Fig. S3). To quantify these observations and make inferences on the nature of the isomers, we measured proportions of all isomers over time in methanol extracts. The 37-min and 38-min isomers comprised 95% of the total yield of LAH in the first measurement of each sample after extraction (recorded from 5 to 14.5 h after extraction) and declined over 12 days at room temperature to 69% of total the LAH measured (Fig. S3). There was no measurable effect of time on the ratio of 37-min to 38-min isomers ($P = 0.32$) (Fig. S3), whereas the ratio of 41-min and 43-min isomers to the 37-min and 38-min isomers increased over time ($P < 0.0001$). These data indicate that the 37-min and 38-min isomers are the forms found naturally in the fungi and that over time in methanol some proportion of them isomerizes to the 41- and 43-min isomers. This pattern is typical of lysergic acid derivatives in protic solvents and is due to keto-enol tautomerization at the carbon atom (customarily called carbon 8) where the amide side branch attaches to the ergoline ring system (marked with an asterisk in Fig. 2) (42, 43).

We observed ergine, the simple amide of lysergic acid, and its stereoisomer erginine in some but not all alkaloid preparations of *Metarhizium* species. Ergine is not a biosynthetic endpoint in itself but rather a hydrolysis product of LAH and some additional ergot alkaloids (42, 44, 45). We measured proportions of ergine/erginine relative to LAH (sum total of all four stereoisomers) over time in *M. brunneum* ARSEF 9354 methanolic extracts (Fig. S3). Consistent with previous observations on its origin, ergine constituted 1% of the total of ergine plus LAH at the time of the initial measurement (which was recorded from 5 to 14.5 h after extraction) and that proportion increased over time to 17% after 12 days of the extracts incubating at room temperature.

When spore suspensions of 26 isolates representing nine species of *Metarhizium* were inoculated into larvae of *G. mellonella*, ergot alkaloid accumulation varied by fungal species (Table 1). Inoculated insects died 2 to 3 days after inoculation and were analyzed 7 to 10 days after inoculation by HPLC. No control insects (inoculated with a spore-free solution) contained ergot alkaloids. Among isolates from the former *M. anisopliae* species complex (i.e., the clade containing *M. anisopliae*, *M. brunneum*, *M. robertsii*, and *M. pingshaense*) (46), 14 of 16 tested isolates accumulated ergot alkaloids. For the five species (10 isolates total) tested outside that clade, ergot alkaloids were detected in only *M. flavoviride* and *M. acridum*. No isolates of *M. album* (including the sequenced isolate ARSEF 1941, which was tested here) or *M. guizhouense* accumulated ergot alkaloids in insects, despite the reference genome for each of these species (37) containing the requisite cluster of genes. Both tested isolates of *M. majus* lacked ergot alkaloids, consistent with the genome of *M. majus* ARSEF 297 as described by Hu et al. (37) (not tested for alkaloids here) containing only a partial ergot alkaloid synthesis cluster. The profile of ergot alkaloids produced by *M. acridum*, *M. flavoviride*, and *M. robertsii* (species that had not produced ergot alkaloids in culture) was like that of *M. anisopliae* and *M. brunneum* in containing LAH, ergonovine, and ergine.

The mean molarity of LAH in *M. brunneum* ARSEF 9354-inoculated larvae was 154 μM ($\pm$ 58 μM standard error of the mean [SE]), approximately 200-fold greater than the concentration that accumulated in SYE cultures of that same isolate of *M. brunneum*. A time course of alkaloid accumulation in *G. mellonella* larvae infected with *M.*

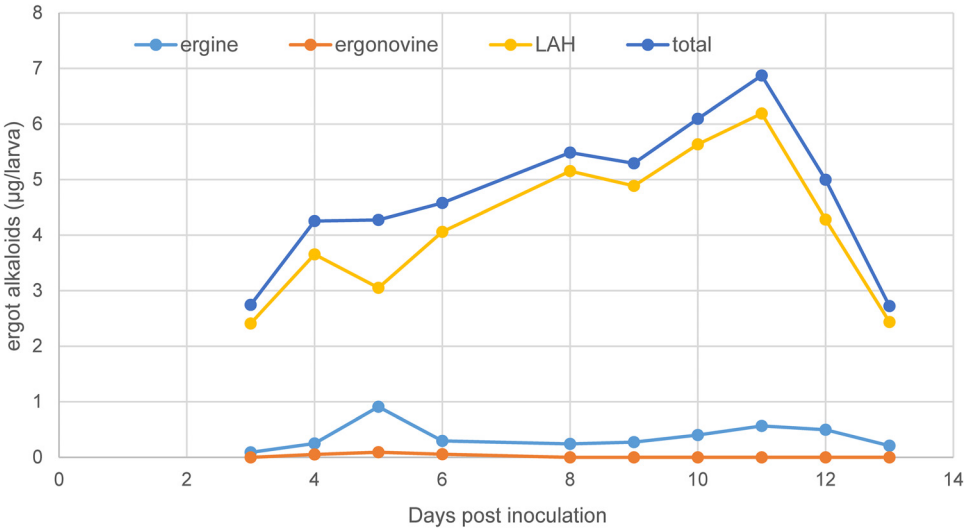

FIG 5 Mean accumulation of ergot alkaloids over time in larvae ($n = 4$) infected with *M. brunneum* ARSEF 9354. Insects died 2 to 3 days after inoculation. Relative proportion of ergine to LAH at day 5 indicates extra hydrolysis on samples from that date. Data are expressed as micrograms per larva as opposed to micrograms/mass of larva, because larval mass changed significantly over time as dead larvae dehydrated.

brunneum ARSEF 9354 showed that ergot alkaloids were first detected at day three postinoculation and peaked at day 11 before declining in concentration (Fig. 5). Inoculation of larvae that were killed by freezing at −20°C and thawing or by prolonged exposure to N_2 gas prior to injection with *M. brunneum* ARSEF 9354 conidia resulted in a significant reduction ($P < 0.05$) in the amount of ergot alkaloids produced per mass of fungus as estimated by fungus-associated ergosterol content in infected larvae (Fig. 6).

Several brood V and brood VIII periodical cicadas (*Magicicada* spp.) naturally infected with a *Metarhizium* species were analyzed and found to contain a profile of ergot alkaloids typical of that observed in infected *G. mellonella* (Fig. S4), though with a higher proportion of ergine to LAH, perhaps due to the unknown and uncontrolled duration and conditions between infection and analysis. This observation indicates that adult insects representing a different order from *G. mellonella* (Hemiptera as opposed to Lepidoptera) are also suitable hosts for ergot alkaloid accumulation.

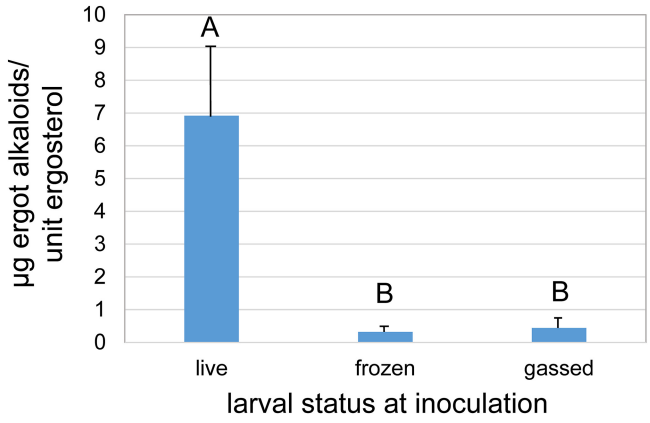

FIG 6 Mean accumulation of ergot alkaloids (sum of LAH, ergine, and ergonovine) in larvae alive at the time of inoculation compared to larvae killed prior to inoculation by freezing or N_2 gas. Treatments marked with different letters were significantly different in a Tukey's test ($P < 0.05$). Error bars represent standard error of the mean ($n = 5$).

TABLE 2 Numbers of compounds[d] that varied between *Metarhizium* species-colonized corn roots compared to uninoculated corn roots by a minimum two log-fold difference[e]

Response in corn roots	Positive mode[a]	Negative mode[a]	Total
Up in *M.b.*[b]; up in *M.f.*[c]	11	28	39
Up in *M.b.*; unchanged in *M.f.*	2	12	14
Up in *M.b.*; down in *M.f.*	0	2	2
Down in *M.b.*; up in *M.f.*	0	0	0
Down in *M.b.*; unchanged in *M.f.*	1	1	2
Down in *M.b.*; down in *M.f.*	1	2	3
Unchanged in *M.b.*; up in *M.f.*	2	0	2
Unchanged in *M.b.*; down in *M.f.*	4	0	4

[a]Ionization mode on mass spectrometer.
[b]*M.b.*, corn roots infected with *M. brunneum* ARSEF 9354.
[c]*M.f.*, corn roots infected with *M. flavoviride* BC 1163.
[d]Detailed list of compounds can be found in Table S1 in the supplemental material.
[e]$P < 0.05$; $n = 3$ biological replicates.

Lack of ergot alkaloid accumulation in corn and bean seedlings inoculated with *Metarhizium* species. *Metarhizium brunneum* is a common rhizosphere inhabitant (21, 47–50) and has been demonstrated to colonize corn roots and increase plant growth under experimental conditions (51). *Metarhizium flavoviride* also has been associated with roots (48, 49). Neither *M. brunneum* nor *M. flavoviride* accumulated ergot alkaloids when inoculated onto young seedlings of bean (*Phaseolus vulgaris*) or corn (*Zea mays*) grown on filter paper. Tissues samples found to be negative for ergot alkaloids when analyzed by HPLC with fluorescence detection included roots, hypocotyls, cotyledons, and first leaf tissues. Fungal mycelia were visible on the surface of the exposed root tissues of these filter paper-grown seedlings, indicating successful colonization (Fig. S5). To provide additional evidence of fungal colonization, a global metabolomics analysis was performed on three biological replicates each of *M. brunneum* ARSEF 9354-inoculated corn roots, *M. flavoviride* BC1163-inoculated roots, and non-inoculated corn roots. The data showed that corn roots treated with *M. brunneum* or *M. flavoviride* had altered accumulation of 66 metabolites compared to control plants that had not been treated with the fungi (Table 2; Table S1). Noteworthy among the compounds that could be identified on the basis of mass spectral fragmentation was the plant hormone γ-aminobutyric acid, which accumulated 20 log-fold higher in *M. brunneum* ARSEF 9354-infected roots and 18 log-fold higher in *M. flavoviride*-infected roots compared to the untreated roots ($P = 0.0002$). These observations indicate that the seedlings were in fact colonized by the fungi, however the plant tissue did not contain ergot alkaloids.

DISCUSSION

Our data show that several *Metarhizium* species produce ergot alkaloids in a condition-dependent manner, an observation that has agricultural and pharmaceutical implications. Only isolates of *M. brunneum* and *M. anisopliae* accumulated alkaloids in culture, even though three other species were later shown to accumulate ergot alkaloids in insects. These observations indicate that regulation of expression of the ergot alkaloid pathway genes differs in these fungi. The lack of accumulation of ergot alkaloids in plants by *M. brunneum* ARSEF 9354, which produced ergot alkaloids in culture and abundant ergot alkaloids in insects, demonstrates further substrate-specific regulation of accumulation of these alkaloids. The increased accumulation when insect larvae were alive at the time of inoculation indicates a further level of regulation and suggests a role for ergot alkaloids in insect colonization.

All ergot alkaloid-positive species of *Metarhizium* produced lysergic acid amides: most abundantly LAH (along with its hydrolysis product ergine) and lesser quantities of ergonovine. LAH occurs in four stereoisomers as documented in previous work with *Claviceps pasapali* (42). The four stereoisomers result from all combinations arising from two chiral carbons: one at the attachment point of the amide side chain to the lysergic acid ring (i.e., lysergic versus isolysergic forms typical of all lysergic acid derivatives) and

the other from L-Ala-derived versus D-Ala-derived hydroxylamide side chains. The relative proportions of stereoisomers derived from the attachment of the amide side chain to lysergic acid change over time due to keto-enol tautomerization in protic solvents (42, 43), whereas the stereoisomers derived from the conformation of alanine have no mechanism to change over time. For these reasons, we hypothesize that the 37-min and 38-min isomers represent the L-Ala-derived and D-Ala-derived isomers in the natural lysergic acid conformation and that they form when LAH is synthesized and subsequently have no mechanism to isomerize between D-Ala-derived and L-Ala-derived forms after synthesis. Some proportion of those initial forms of LAH (i.e., the 37-min and 38-min isomers) subsequently isomerize from natural lysergic acid conformation to isolysergic acid conformation (i.e., the 42-min and 43-min isomers) after extraction in protic solvents as previously documented for all lysergic acid derivatives (42, 43). Our proposed assignment of stereoisomers to peak pairs observed in our chromatograms agrees with the relative abundances observed by Flieger et al. (42) in their studies of *C. paspali*. Small quantities of ergonovine also were detected in most extracts of *Metarhizium* species. Ergonovine is another amide of lysergic acid and is derived by reduction of the carbonyl group of lysergyl-alanine to a primary alcohol by the enzyme LpsC (35). In our preparations from *Metarhizium* species, ergonovine was typically present at less than 2% of the concentration of LAH.

LAH has been detected previously in *C. paspali*, a pathogen of *Paspalum* species (Poaceae), and in several *Periglandula* species that grow as seed-transmitted and unculturable symbionts of morning glories (Convolvulaceae) (4, 39). The discovery of LAH in *Metarhizium* species, which can be genetically manipulated, presents an opportunity to functionally analyze genes required for LAH synthesis. The correlation of genes *easO* and *easP* with LAH producers (Fig. 1) indicates a potential role for these genes in later stages of LAH biosynthesis. *Metarhizium brunneum* may provide a more tractable system for functional analysis of these genes than the non-culturable *Periglandula* spp. or the plant pathogen *C. paspali*. Interestingly, *M. brunneum* ARSEF 9354 secreted the vast majority of its ergot alkaloids into the culture medium. In this way it differs from other ergot alkaloid-producing fungi, such as *N. fumigata* and *Claviceps purpurea*, which retain much of their ergot alkaloids in their mycelia, complicating extraction and purification. The ability of *Metarhizium* species to secrete ergot alkaloids would be an advantage for pharmaceutical production and development.

The lack of ergot alkaloids in conidia (typically used as inoculum for biocontrol) and in *Metarhizium* sp.-infected plants indicates that the ergot alkaloid biosynthesis capacity of these fungi should not present complications for the use of the fungi as biocontrol agents. Moreover, our metabolomic data showed differences in roots inoculated with *M. brunneum* and *M. flavoviride* indicating colonization of plant roots, but the association did not induce accumulation of ergot alkaloids. Colonization of corn was supported by metabolomics data, including the demonstration of increased quantities of the plant hormone γ-aminobutyric acid. These data show that some key metabolites such as γ-aminobutyric acid are upregulated, whereas others are downregulated in roots of corn that were treated with *M. brunneum* or *M. flavoviride*. Other metabolites varied between corn roots that were treated with different species of *Metarhizium*, indicating that corn responds differently to different species of *Metarhizium*. The lack of ergot alkaloids in infected plant tissue indicates the presence of *Metarhizium* species on the roots or in the rhizosphere of plants is not of concern from a safety perspective.

Metarhizium species are commonly used as mycoinsecticides to control pest insects. Data from inoculations of dead versus living *G. mellonella* larvae indicate that the living insect provides an environment that induces ergot alkaloid synthesis. Whether or not the ergot alkaloids play a direct role in killing of insects has not yet been established. Future genetic studies, such as analyses of planned gene knockout mutants lacking ergot alkaloids, should provide material to facilitate tests of whether ergot alkaloids play a role in insect pathogenesis.

MATERIALS AND METHODS

Fungi and culture conditions. Isolates of *Metarhizium* species studied and their substrates of origin are listed in Table 1. Isolates with ARSEF numbers were obtained from the United States Department of Agriculture-ARS Collection of Entomopathogenic Fungal Cultures (Ithaca, NY). *Metarhizium guizhouense* CBS 258.90 was obtained from the Westerdijk Fungal Biodiversity Institute (Utrecht, The Netherlands). *Metarhizium flavoviride* BC1163 and *M. anisopliae* BC1203 were isolated from the millipede *Brachycybe lecontii* collected in Arkansas and Oklahoma, respectively (52). Millipede-associated *Metarhizium* species were confirmed by sequencing the ribosomal internal transcribed spacer (ITS) (deposited as GenBank accessions MN963924 and MT040791) and a portion of the 28S rRNA gene (GenBank accession MN963928) genes. During our work with the isolate initially supplied as *M. anisopliae* ARSEF 9354, we noticed several discrepancies between the published *M. anisopliae* sequences and fragments of the ARSEF 9354 isolate we sequenced, and these observations spurred further research into the identity of this isolate. We amplified and sequenced the translation elongation factor 1α (EF1α) (GenBank accession MH712765) and ITS (GenBank accession MH711991) alleles from ARSEF 9354 and compared them to the corresponding alleles in the genomes of previously sequenced *Metarhizium* species. The best matches were to the genome of *M. brunneum* ARSEF 3297 (99.7% EF1α, 100% ITS), with our sequences providing a lower match to *M. anisopliae* ARSEF 549 (97.9% EF1α, 99% ITS). Query coverage was 100% in each of these comparisons. Based on these findings we concluded that ARSEF 9354 is an isolate of *M. brunneum*.

Two isolates of *M. brunneum* (ARSEF 9354 and ARSEF 3826) and one isolate of *M. flavoviride* (BC1163) were grown on three different culture media: sucrose-yeast extract agar (SYE), corn meal agar (CMA), and malt extract agar (MEA). Once SYE was determined to be the best of these media for promoting ergot alkaloid accumulation, isolates of additional *Metarhizium* species were tested exclusively on this medium. SYE contained (per liter) 20 g sucrose, 10 g yeast extract, 1 g of magnesium sulfate-heptahydrate, 2 ml of trace element solution (53), and 15 g agar. CMA contained (per liter) 2 g corn meal and 15 g agar. MEA contained (per liter) 6 g malt extract, 1.8 g maltose, 6 g glucose, 1.2 g yeast extract, and 15 g agar. All cultures were grown at room temperature (22 to 24°C) with ambient lighting for at least 7 days before testing for ergot alkaloids.

Ergot alkaloid extraction and analysis. Samples of approximately 400 μl (and consisting of colonized agar, mycelia, and conidia) were cut from colonized culture medium with the broad end of a 1,000-μl pipet tip 7 to 10 days after inoculation. Samples were suspended in 400 μl of 100% methanol in a 1.5-ml microcentrifuge tube, vortexed, and rotated end over end on a Glas-Col (Terre Haute, IN) minirotator at 40 rpm for 20 min. Methanol extracts were analyzed by HPLC with fluorescence detection based on the methods described in Panaccione et al. (43). The HPLC apparatus consisted of a Waters model 600 pump controller with an in-line degasser, a Waters model 717plus autosampler, and a Rainin (Woburn, MA) FI2 fluorescence detector set at excitation and emission wavelengths of 310 nm and 410 nm (to detect lysergic acid derivatives), respectively, and a Linear 304 fluorescence detector with excitation at 272 nm and emission monitored at 372 nm (to detect simpler clavine-type ergot alkaloids and dihydroergot alkaloids). Approximate concentrations of LAH, ergonovine, and ergine were estimated by comparing peak area to an external standard curve prepared from ergotamine, which contains the same lysergic acid fluorophore, and adjusting for relative masses of analytes and standard.

The identities of the peak corresponding to ergonovine was determined by fragmentation compared to the ergonovine analytical standard obtained from Sigma (St. Louis, MO) in LC-MS assays. The identities of peaks corresponding to ergine and lysergic acid α-hydroxyethylamide (LAH) were determined by fragmenting analytes from *M. brunneum* compared to those obtained from *Periglandula* species-infected *Ipomoea leptophylla*, which had previously been demonstrated to contain these lysergic acid amides (39). LC-MS was performed in positive ion mode as described by Ryan et al. (54), and high-resolution data were obtained by injecting methanol extracts into an Accela 1290 UHPLC coupled to a Thermo Fisher Scientific Q Exactive mass spectrometer operated in positive polarity mode with a data-dependent acquisition scheme.

To assess secretion of ergot alkaloids from *M. brunneum* ARSEF 9354, cultures were grown from 50,000 conidia in 5 ml of SYE broth (as described above, however without the addition of agar) at room temperature in petri dishes that were 3.5 cm in diameter for 7 days. The entirety of an individual culture (hyphae, conidia, and medium) was filtered through a preweighed 0.2-μm nylon filter under vacuum to separate the fungal mycelia and spores from the culture medium. The harvested solid material was dried, weighed, and a measured sample was analyzed for ergot alkaloids (as described above) to determine the total amount of ergot alkaloids in the solid phase of that culture. The volume of the liquid phase was measured, a subsample was analyzed for ergot alkaloids as described above, and the total amount of ergot alkaloids in the liquid phase was calculated.

To assess whether ergot alkaloids were contained in or on conidia of *M. brunneum*, conidia were collected from the inner surface of the lids of inverted petri dishes with methanol. After incubation at room temperature for 1 h, the mass of conidia extracted was determined by collecting conidia on a preweighed 0.2-μm spin filter and weighing the filter with collected conidia after drying. The clarified methanol extracts were analyzed by fluorescence HPLC as described above.

Ergosterol was measured with a method adapted from Zill et al. (55). Methanol extracts were analyzed by reversed-phase separation with isocratic elution in 99% methanol on an Agilent 1100 HPLC instrument (Agilent Technologies, Santa Clara, CA). Ergosterol was monitored with the multiwavelength detector set at 282 nm and identified by comparison to an authentic standard (Sigma-Aldrich).

Statistical comparisons of ergot alkaloid data were made with JMP (SAS, Cary, NC). Data were checked for unequal variances with a Brown-Forsythe test, and means were compared with ANOVA and a *post hoc* Tukey's test.

Insect inoculation assays. Larvae of *Galleria mellonella*, commonly referred to as wax worms, were purchased from New York Worms (Glen Cove, NY), and individuals were selected for infection based on appearance (adequate size and lack of black coloration, which may indicate prior infection) and mobility. Spore suspensions contained 40,000 conidia/μl in a modified phosphate-buffered saline (PBS) solution (pH 7.4) containing 9 mM Na_2PO_4, 1.6 mM KH_2PO_4, 123 mM NaCl, 0.01% wt/vol Tween 20, and 10 μg/ml rifampin. Each larva was injected with 20 μl of spore solution via an insulin syringe with a 29.5-gauge needle according to methods described by Panaccione and Arnold (56). Larvae in control groups were inoculated in the same manner with 20 μl of modified PBS solution lacking conidia. Inoculated larvae were incubated at room temperature. Once the larvae died, they were incubated routinely for 7 days at room temperature under ambient lighting. In a time course study, larval samples ($n = 4$) were extracted daily for up to 13 days. Their weights were then recorded and 1 ml of methanol and ten 3-mm glass beads were added. The larvae were homogenized in a Fastprep 120 bead beater (Bio101; Carlsbad, CA), rotated for 20 min on the minirotator, clarified by centrifugation, and analyzed via HPLC with fluorescence detection (as described above).

Plant inoculation assays. Corn (*Zea mays*) and bean (*Phaseolus vulgaris*) seeds were sterilized in an aqueous mixture of 20% bleach and 0.01% Tween 20 for 20 min rotating at 40 rpm on the Glas-Col (Terre Haute, IN) minirotator. Seeds were rinsed with sterilized distilled water repeatedly and placed in petri dishes containing filter paper saturated with Hoagland's solution (57). Seeds were incubated under light that ranged from 50 to 60 μmol/m^2/sec at room temperature. Seeds were illuminated for 16 h/day, and Hoagland's solution was replenished as needed to maintain moisture. Roots were inoculated when the emerged radicle reached a length greater than that of the seed, and uninoculated roots were left as a control treatment.

Metarhizium flavoviride BC1163 and *M. brunneum* ARSEF 9354 were cultured for 7 days on malt extract agar prior to inoculation. Both cultures were then used to create suspensions of 200,000 conidia per ml in 0.01% Tween 20. The concentration of spores in each sample was calculated with the aid of a hemocytometer. Spore suspensions were administered directly to the roots of each plant via a micropipette.

Plant samples were grown on filter paper moistened with Hoagland's solution and under light and temperature conditions as described above for 14 days, then were divided into roots, hypocotyls, cotyledons, and leaves. Tissues were dried and dry weight was recorded. Weighed subsamples of respective tissues were placed into 2-ml tubes along with 10 glass beads and 1 ml of methanol. Plant samples (50 mg dry weight per ml of methanol) were homogenized by bead beating as described above for insect samples. The samples were then clarified by centrifugation, and 20 μl of sample were injected into the fluorescence HPLC system described above to assay for ergot alkaloids.

Metabolomic analyses of control (untreated) corn roots, corn roots inoculated with *M. brunneum* ARSEF 9354, and corn roots inoculated with *M. flavoviride* BC1163 ($n = 3$ biological replicates for each) were performed in two stages. The first stage involved discovery of parent ions (analyzed in both positive ion mode and negative ion mode) that varied in abundance among treatments. These data were collected on an Agilent 6530 QToF at Protea Biosciences (Morgantown, WV). Metabolite abundance was compared in one-way ANOVAs and considered to be different if abundances varied by a log fold change factor of two ($P < 0.05$). In the second stage, identities of compounds yielding parent ions that varied in abundance among treatments in stage 1 were obtained in data-dependent assays in which tandem mass spectrometry (MS-MS) scans were compared in Compound Discover software (Thermo Fisher Scientific, Waltham, MA) to libraries of MS-MS data in KEGGS, BioCyc, and Metlin metabolite databases (mass tolerance 10 ppm). The data-dependent analyses were performed on a Thermo Q Exactive MS at the Shared Research Facilities at WVU.

Data availability. Millipede-associated *Metarhizium* species were confirmed by sequencing the ribosomal internal transcribed spacer (ITS) (deposited as GenBank accessions MN963924 and MT040791) and a portion of the 28S rRNA gene (GenBank accession MN963928) genes. During our work with the isolate initially supplied as *M. anisopliae* ARSEF 9354, we noticed several discrepancies between the published *M. anisopliae* sequences and fragments of the ARSEF 9354 isolate we sequenced, and these observations spurred further research into the identity of this isolate. We amplified and sequenced the translation elongation factor 1α (EF1α) (GenBank accession MH712765) and ITS (GenBank accession MH711991) alleles from ARSEF 9354.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.7 MB.

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REFERENCES

1. Klotz J. 2015. Activities and effects of ergot alkaloids on livestock physiology and production. Toxins (Basel) 7:2801–2821. https://doi.org/10.3390/toxins7082801.
2. Craig AM, Klotz JL, Duringer JM. 2015. Cases of ergotism in livestock and associated ergot alkaloid concentrations in feed. Front Chem 3:8. https://doi.org/10.3389/fchem.2015.00008.
3. Haarmann T, Rolke Y, Giesbert S, Tudzynski P. 2009. Ergot: from witchcraft to biotechnology. Mol Plant Pathol 10:563–577. https://doi.org/10.1111/j.1364-3703.2009.00548.x.
4. Florea S, Panaccione DG, Schardl CL. 2017. Ergot alkaloids of the Clavicipitaceae. Phytopathology 107:504–518. https://doi.org/10.1094/PHYTO-12-16-0435-RVW.
5. Donnet A, Braunstein D, Pradel V, Sciortino V, Allaria-Lapierre V, Micallef J, Lanteri-Minet M. 2016. Ergot use and overuse: a pharmacoepidemiology retrospective cohort study. Headache 56:547–554. https://doi.org/10.1111/head.12776.
6. Tfelt-Hansen PC. 2013. Triptans and ergot alkaloids in the acute treatment of migraine: similarities and differences. Expert Rev Neurother 13:961–963. https://doi.org/10.1586/14737175.2013.832851.
7. Baskys A, Hou AC. 2007. Vascular dementia: pharmacological treatment approaches and perspectives. Clin Interv Aging 2:327–335.
8. Perez-Lloret S, Rascol O. 2010. Dopamine receptor agonists for the treatment of early or advanced Parkinson's disease. CNS Drugs 24:941–968. https://doi.org/10.2165/11537810-000000000-00000.
9. Robinson SL, Panaccione DG. 2015. Diversification of ergot alkaloids in natural and modified fungi. Toxins (Basel) 7:201–218. https://doi.org/10.3390/toxins7010201.
10. Saikkonen K, Young CA, Helander M, Schardl CL. 2016. Endophytic *Epichloë* species and their grass hosts: from evolution to applications. Plant Mol Biol 90:665–675. https://doi.org/10.1007/s11103-015-0399-6.
11. Schardl CL, Florea S, Pan J, Nagabhyru P, Bec S, Calie PJ. 2013. The epichloae: alkaloid diversity and roles in symbiosis with grasses. Curr Opin Plant Biol 16:480–488. https://doi.org/10.1016/j.pbi.2013.06.012.
12. Panaccione DG, Cipoletti JR, Sedlock AB, Blemings KP, Schardl CL, Machado C, Seidel GE. 2006. Effects of ergot alkaloids on food preference and satiety in rabbits, as assessed with gene knockout endophytes in perennial ryegrass (*Lolium perenne*). J Agric Food Chem 54:4582–4587. https://doi.org/10.1021/jf060626u.
13. Parish JA, McCann MA, Watson RH, Hoveland CS, Hawkins LL, Hill NS, Bouton JH. 2003. Use of nonergot alkaloid-producing endophytes for alleviating tall fescue toxicosis in sheep. J Anim Sci 81:1316–1322. https://doi.org/10.2527/2003.8151316x.
14. Parish JA, McCann MA, Watson RH, Paiva NN, Hoveland CS, Parks AH, Upchurch BL, Hill NS, Bouton JH. 2003. Use of nonergot alkaloid-producing endophytes for alleviating tall fescue toxicosis in stocker cattle. J Anim Sci 81:2856–2868. https://doi.org/10.2527/2003.81112856x.
15. Popay AJ, Jensen JG, Cooper BM. 2005. The effect of non-toxic endophytes in tall fescue on two major insect pests. Proc NZ Grassl Assoc 67:169–173.
16. Potter DA, Stokes JT, Redmond CT, Schardl CL, Panaccione DG. 2008. Contribution of ergot alkaloids to suppression of a grass-feeding caterpillar assessed with gene-knockout endophytes in perennial ryegrass. Entomol Exp Appl 126:138–147. https://doi.org/10.1111/j.1570-7458.2007.00650.x.
17. Kaur N, Cooper WR, Duringer JM, Badillo-Vargas IE, Esparza-Díaz G, Rashed A, Horton DR. 2018. Survival and development of potato psyllid (Hemiptera: Triozidae) on Convolvulaceae: effects of a plant-fungus symbiosis (*Periglandula*). PLoS One 13:e0201506. https://doi.org/10.1371/journal.pone.0201506.
18. Sasan RK, Bidochka MJ. 2012. The insect-pathogenic fungus *Metarhizium robertsii* (Clavicipitaceae) is also an endophyte that stimulates plant root development. Am J Bot 99:101–117. https://doi.org/10.3732/ajb.1100136.
19. Wang S, Fang W, Wang C, St. Leger RJ. 2011. Insertion of an esterase gene into a specific locust pathogen (*Metarhizium acridum*) enables it to infect caterpillars. PLoS Pathog 7:e1002097. https://doi.org/10.1371/journal.ppat.1002097.
20. Hu G, St. Leger RJ. 2002. Field studies using a recombinant mycoinsecticide (*Metarhizium anisopliae*) reveal that it is rhizosphere competent. Appl Environ Microbiol 68:6383–6387. https://doi.org/10.1128/aem.68.12.6383-6387.2002.
21. Wyrebek M, Huber C, Sasan RK, Bidochka MJ. 2011. Three sympatrically occurring species of *Metarhizium* show plant rhizosphere specificity. Microbiology 157:2904–2911. https://doi.org/10.1099/mic.0.051102-0.
22. de Faria MR, Wraight SP. 2007. Mycoinsecticides and mycoacaricides: a comprehensive list with worldwide coverage and international classification of formulation types. Biol Control 43:237–256. https://doi.org/10.1016/j.biocontrol.2007.08.001.
23. Iwanicki NS, Pereira A, Botelho ABRZ, Rezende JM, de Andrade Moral R, Zucchi MI, Delalibera I, Jr. 2019. Monitoring of the field application of *Metarhizium anisopliae* in Brazil revealed high molecular diversity of *Metarhizium* spp. in insects, soil and sugarcane roots. Sci Rep 9:4443. https://doi.org/10.1038/s41598-019-38594-8.
24. Langewald J, Kooyman C. 2007. Green Muscle, a fungal biopesticide for control of grasshoppers and locusts in Africa, p 311–327. *In* Vincent C, Goettel MS, Lazarovitis G (ed), Biological control, a global perspective. CABI, Oxfordshire, UK.
25. St. Leger RJ, Wang C. 2010. Genetic engineering of fungal biocontrol agents to achieve greater efficacy against insect pests. Appl Microbiol Biotechnol 85:901–907. https://doi.org/10.1007/s00253-009-2306-z.
26. Lovett B, Bilgo E, Millogo SA, Ouattarra AK, Sare I, Gnambani EJ, Dabire RK, Diabate A, St. Leger RJ. 2019. Transgenic *Metarhizium* rapidly kills mosquitoes in a malaria-endemic region of Burkina Faso. Science 364:894–897. https://doi.org/10.1126/science.aaw8737.
27. St Leger RJ, Joshi L, Bidochka MJ, Roberts DW. 1996. Construction of an improved mycoinsecticide overexpressing a toxic protease. Proc Natl Acad Sci U S A 93:6349–6354. https://doi.org/10.1073/pnas.93.13.6349.
28. Boldo JT, Junges A, Do Amaral KB, Staats CC, Vainstein MH, Schrank A. 2009. Endochitinase CHI2 of the biocontrol fungus *Metarhizium anisopliae* affects its virulence toward the cotton stainer bug *Dysdercus peruvianus*. Curr Genet 55:551–560. https://doi.org/10.1007/s00294-009-0267-5.
29. Duan Z, Shang Y, Gao Q, Zheng P, Wang C. 2009. A phosphoketolase Mpk1 of bacterial origin is adaptively required for full virulence in the insect-pathogenic fungus *Metarhizium anisopliae*. Environ Microbiol 11:2351–2360. https://doi.org/10.1111/j.1462-2920.2009.01961.x.
30. Wang B, Kang Q, Lu Y, Bai L, Wang C. 2012. Unveiling the biosynthetic puzzle of destruxins in *Metarhizium* species. Proc Natl Acad Sci U S A 109:1287–1292. https://doi.org/10.1073/pnas.1115983109.
31. Gao Q, Jin K, Ying SH, Zhang Y, Xiao G, Shang Y, Duan Z, Hu X, Xie XQ, Zhou G, Peng G, Luo Z, Huang W, Wang B, Fang W, Wang S, Zhong Y, Ma LJ, St. Leger RJ, Zhao GP, Pei Y, Feng MG, Xia Y, Wang C. 2011. Genome sequencing and comparative transcriptomics of the model entomopathogenic fungi *Metarhizium anisopliae* and *M. acridum*. PLoS Genet 7:e1001264. https://doi.org/10.1371/journal.pgen.1001264.
32. Schardl CL, Young CA, Hesse U, Amyotte SG, Andreeva K, Calie PJ, Fleetwood DJ, Haws DC, Moore N, Oeser B, Panaccione DG, Schweri KK, Voisey CR, Farman ML, Jaromczyk JW, Roe BA, O'Sullivan DM, Scott B, Tudzynski P, An Z, Arnaoudova EG, Bullock CT, Charlton ND, Chen L, Cox M, Dinkins RD, Florea S, Glenn AE, Gordon A, Güldener U, Harris DR, Hollin W, Jaromczyk J, Johnson RD, Khan AK, Leistner E, Leuchtmann A, Li C, Liu J, Liu J, Liu M, Mace W, Machado C, Nagabhyru P, Pan J, Schmid J, Sugawara K, Steiner U, Takach JE, Tanaka E, Webb JS, Wilson EV, Wiseman JL, Yoshida R, Zeng Z. 2013. Plant-symbiotic fungi as chemical engineers: multi-genome analysis of the Clavicipitaceae reveals dynamics of alkaloid loci. PLoS Genet 9:e1003323. https://doi.org/10.1371/journal.pgen.1003323.
33. Schardl CL, Young CA, Pan J, Florea S, Takach JE, Panaccione DG, Farman ML, Webb JS, Jaromczyk J, Charlton ND, Nagabhyru P, Chen L, Shi C, Leuchtmann A. 2013. Currencies of mutualisms: sources of alkaloid genes in vertically transmitted epichloae. Toxins (Basel) 5:1064–1088. https://doi.org/10.3390/toxins5061064.
34. Correia T, Grammel N, Ortel I, Keller U, Tudzynski P. 2003. Molecular cloning and analysis of the ergopeptine assembly system in the ergot

fungus *Claviceps purpurea*. Chem Biol 10:1281–1292. https://doi.org/10.1016/j.chembiol.2003.11.013.
35. Ortel I, Keller U. 2009. Combinatorial assembly of simple and complex D-lysergic acid alkaloid peptide classes in the ergot fungus *Claviceps purpurea*. J Biol Chem 284:6650–6660. https://doi.org/10.1074/jbc.M807168200.
36. Pattemore JA, Hane JK, Williams AH, Wilson BAL, Stodart BJ, Ash GJ. 2014. The genome sequence of the biocontrol fungus *Metarhizium anisopliae* and comparative genomics of *Metarhizium* species. BMC Genomics 15:660. https://doi.org/10.1186/1471-2164-15-660.
37. Hu X, Xiao G, Zheng P, Shang Y, Su Y, Zhang X, Liu X, Zhan S, St. Leger RJ, Wang C. 2014. Trajectory and genomic determinants of fungal-pathogen speciation and host adaptation. Proc Natl Acad Sci U S A 111:16796–16801. https://doi.org/10.1073/pnas.1412662111.
38. Shang Y, Xiao G, Zheng P, Cen K, Zhan S, Wang C. 2016. Divergent and convergent evolution of fungal pathogenicity. Genome Biol Evol 8:1374–1387. https://doi.org/10.1093/gbe/evw082.
39. Beaulieu WT, Panaccione DG, Ryan KL, Kaonongbua W, Clay K. 2015. Phylogenetic and chemotypic diversity of *Periglandula* species in eight new morning glory hosts (Convolvulaceae). Mycologia 107:667–678. https://doi.org/10.3852/14-239.
40. Mulinti P, Allen NA, Coyle CM, Gravelat FN, Sheppard DC, Panaccione DG. 2014. Accumulation of ergot alkaloids during conidiophore development in *Aspergillus fumigatus*. Curr Microbiol 68:1–5. https://doi.org/10.1007/s00284-013-0434-2.
41. Panaccione DG, Coyle CM. 2005. Abundant respirable ergot alkaloids from the common airborne fungus *Aspergillus fumigatus*. Appl Environ Microbiol 71:3106–3111. https://doi.org/10.1128/AEM.71.6.3106-3111.2005.
42. Flieger M, Sedmera P, Vokoun J, Ricicova A, Rehacek Z. 1982. Separation of four isomers of lysergic acid α-hydroxyethylamide by liquid chromatography and their spectroscopic identification. J Chromatogr 236:453–459. https://doi.org/10.1016/S0021-9673(00)84895-5.
43. Panaccione DG, Ryan KL, Schardl CL, Florea S. 2012. Analysis and modification of ergot alkaloid profiles in fungi. Methods Enzymol 515:267–290. https://doi.org/10.1016/B978-0-12-394290-6.00012-4.
44. Ramstad E. 1968. Chemistry of alkaloid formation in ergot. Lloydia 31:327–341.
45. Kleinerova E, Kybal J. 1973. Ergot alkaloids IV. Contribution to the biosynthesis of lysergic acid amides. Folia Microbiol (Praha) 18:390–392. https://doi.org/10.1007/BF02875934.
46. Rehner SA, Kepler RM. 2017. Species limits, phylogeography and reproductive mode in the *Metarhizium anisopliae* complex. J Invertebr Pathol 148:60–66. https://doi.org/10.1016/j.jip.2017.05.008.
47. Kepler RM, Ugine TA, Maul JE, Cavigelli MA, Rehner SA. 2015. Community composition and population genetics of insect pathogenic fungi in the genus *Metarhizium* from soils of a long-term agricultural research system. Environ Microbiol 17:2791–2804. https://doi.org/10.1111/1462-2920.12778.
48. Keyser CA, De Fine Licht HH, Steinwender BM, Meyling NV. 2015. Diversity within the entomopathogenic fungal species *Metarhizium flavoviride* associated with agricultural crops in Denmark. BMC Microbiol 15:249. https://doi.org/10.1186/s12866-015-0589-z.
49. Fisher JJ, Rehner SA, Bruck DJ. 2011. Diversity of rhizosphere associated entomopathogenic fungi of perennial herbs, shrubs and coniferous trees. J Invertebr Pathol 106:289–295. https://doi.org/10.1016/j.jip.2010.11.001.
50. Hu S, Bidochka MJ. 2019. Root colonization by endophytic insect-pathogenic fungi. J Appl Microbiol https://doi.org/10.1111/jam.14503.
51. Liao X, O'Brien TR, Fang W, St. Leger RJ. 2014. The plant beneficial effects of *Metarhizium* species correlate with their association with roots. Appl Microbiol Biotechnol 98:7089–7096. https://doi.org/10.1007/s00253-014-5788-2.
52. Macias AM, Marek PE, Morrissey EM, Brewer MS, Short DPG, Stauder CM, Wickert KL, Berger MC, Metheny AM, Stajich JE, Boyce G, Rio RVM, Panaccione DG, Wong V, Jones TH, Kasson MT. 2019. Diversity and function of fungi associated with the fungivorous millipede, *Brachycybe lecontii*. Fungal Ecol 41:187–197. https://doi.org/10.1016/j.funeco.2019.06.006.
53. Hutner SH, Provasoli L, Schatz A, Haskins CP. 1950. Some approaches to the study of the role of metals in the metabolism of microorganisms. Am Philos Soc Proc 94:152–170.
54. Ryan KL, Akhmedov NG, Panaccione DG. 2015. Identification and structural elucidation of ergotryptamine, a new ergot alkaloid produced by genetically modified *Aspergillus nidulans* and natural isolates of *Epichloî* species. J Agric Food Chem 63:61–67. https://doi.org/10.1021/jf505718x.
55. Zill G, Engelhardt G, Wallnofer PR. 1988. Determination of ergosterol as a measure of fungal growth using Si-60 HPLC. Z Lebensm Unters Forsch 187:246–249. https://doi.org/10.1007/BF01043348.
56. Panaccione DG, Arnold SL. 2017. Ergot alkaloids contribute to virulence in an insect model of invasive. Sci Rep 7:8930. https://doi.org/10.1038/s41598-017-09107-2.
57. Hoagland DR, Arnon DI. 1950. The water-culture method for growing plants without soil, circular 347. California Agricultural Experiment Station, Berkeley, CA.